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Virological Importance of the Protease-Cleavage Site in Human Immunodeficiency Virus Type 1 Nef Is Independent of both Intravirion Processing and CD4 Down-regulation

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The HIV-1 Nef protein is present within the virion and is processed there by the viral protease. Mutational analysis indicated that residues 54–60 in HIV-1 Nef were required for intravirion cleavage. When viruses were produced using T cell lines or primary lymphoblasts, these residues were also required for optimal viral infectivity. However, substitution of native Nef residues with those of a functional Gag cleavage site demonstrated that intravirion cleavage was insufficient for the virological function of this domain. Furthermore, the importance of certain cleavage site residues to infectivity was conditional on the producer cell type. In particular, a mutant containing a deletion of residues 54–57 was phenotypically *nef* defective when produced using T cells (CEM, A2.01, or primary lymphoblasts) but was minimally impaired when produced from 293 or HeLa cells. This mutant was cleavage resistant, indicating that proteolytic processing of Nef was dispensable for infectivity enhancement when virions were assembled in certain non-T cells. Residues 54–61 of the cleavage site, including 54–57, were also required for Nef-mediated down-regulation of CD4. However, the surface expression of CD4 on HeLa cells in amounts comparable to that on the surface of primary T lymphoblasts did not create a producer cell environment in which residues 54–57 acquired greater virological importance. Furthermore, these residues were required for optimal infectivity even during virion assembly in T cells (A2.01) that expressed a CD4 molecule that is unable to respond to Nef. These data suggested that in producer T cells, certain cleavage site residues (54–57) contribute to a Nef-mediated virological effect that is unlikely to be linked causally to CD4 down-regulation. Conversely, in the context of 293 cells as viral producers, the Δ54–57 mutant separated genetically down-regulation of CD4 (for which it was defective) from enhancement of infectivity (for which it was functional). Together, these data indicate that the virological function of the cleavage site domain is both independent of intravirion proteolytic processing of Nef and independent of CD4 down-regulation.

INTRODUCTION

The *nef* gene of primate lentiviruses is an important virulence factor in animal models of infection (Kestler *et al.,* 1991). The observation that certain long-term survivors of HIV-1 infection harbor *nef*-defective viral genomes further suggests a critical role for *nef* in human disease (Deacon *et al.,* 1995). *In vitro*, *nef* is required for efficient viral replication in both cultures of immortalized T cells and in primary cultures of peripheral blood mononuclear cells (PBMCs) (Chowers *et al.,* 1994; de Ronde *et al.,* 1992; Miller *et al.,* 1994; Ryan-Graham and Peden, 1995; Spina *et al.,* 1994).

The mechanism by which *nef* enhances viral replication and pathogenesis is not understood. Several functions have been ascribed to the *nef* gene that might account for these properties. First, Nef down-regulates CD4 and MHC class I from the cell surface by enhancing the rate of endocytosis of these receptors from the plasma membrane (Aiken *et al.,* 1994; Garcia *et al.,* 1994; Schwartz *et al.,* 1996). Second, Nef influences certain signal transduction and T cell activation events, possibly as a result of association with cellular kinases via its SH3-binding domain (Baur *et al.,* 1994; Collette *et al.,* 1996; Du *et al.,* 1995; Greenway *et al.,* 1995). Third, expression of Nef within an infected cell increases the infectivity of progeny viral particles by an incompletely characterized mechanism (Chowers *et al.,* 1994; Miller *et al.,* 1994, 1995; Schwartz *et al.,* 1995).

To enhance infectivity, Nef modifies the virion during its production. Provision of Nef *in trans* to genetically *nef*-negative virus during its production restores virion infectivity to wild-type levels and also restores the productivity of the progeny virions to wild-type levels for one subsequent cycle of replication (Miller *et al.,* 1995; Pandori *et al.,* 1996). Furthermore, the finding that *nef*-negative virus is impaired in its ability to synthesize viral DNA in the newly infected cell indicates that the infectivity phenotype is manifest before viral gene expression in the target cell (Aiken and Trono, 1995; Chowers *et al.,* 1995; Schwartz *et al.,* 1995).

Enhancement of infectivity is a phenomenon which is determined during viral assembly in the producer cell,

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and a variety of producer cell types are capable of providing an environment permissive for this function of Nef. Nef-mediated infectivity enhancement does not require viral production by T cells or even by CD4-positive cells; cells such as COS or 293 appear to support efficiently the ability of Nef to enhance virion infectivity (Aiken and Trono, 1995; Miller *et al.,* 1994, 1995). In fact, no producer cell type has yet been identified that fails to support this function of Nef, including cells that express mutant CD4 molecules that cannot respond to Nef or cells that lack MHC class I (Le Gall *et al.,* 1997; Schwartz *et al.,* 1995). These data have led to the conclusion that the effect of Nef on the expression of these cellular receptors is unrelated causally to the virion modification that enhances infectivity.

The biochemical modification of virions induced by Nef that enhances infectivity is not known. Virions assembled in the absence of Nef appear to contain wild-type levels of viral RNA, reverse transcriptase activity, and envelope glycoprotein (Env) (Aiken and Trono, 1995; Chowers *et al.,* 1995; Schwartz *et al.,* 1995). Notably, Nef-mediated enhancement of infectivity does not require specifically HIV-1 Env. Nef enhances the infectivity of HIV-1 pseudotypes containing murine retroviral Env, although the infectivity of pseudotypes containing vesicular stomatitis virus G protein is relatively insensitive to Nef (Aiken, 1997; Aiken and Trono, 1995; Miller *et al.,* 1995).

Recently, analysis of purified virions has revealed that Nef is a virion–protein (Bukovsky *et al.,* 1997; Pandori *et al.,* 1996; Welker *et al.,* 1996). This observation suggests the simple hypothesis that Nef acts to enhance infectivity directly within the virion or after its delivery by the virion to the newly infected target cell. The Nef protein within virions is cleaved specifically by the viral protease, generating two fragments of ~7 kDa and ~20 kDa (Bukovsky *et al.,* 1997; Pandori *et al.,* 1996; Welker *et al.,* 1996). Cleavage of recombinant $Nef_{HIV\text{-}NL43}$ protein by the HIV-1 protease in cell-free reactions occurs between amino acid residues W57 and L58 (Freund *et al.,* 1994b; Gaedigk-Nitschko *et al.,* 1995). These residues are central to the sequence ACAWLEAQ, which is highly conserved among HIV-1 isolates. With the exception of the amino-terminal alanine that occurred as aspartate in ~50% of *nef* genes, these residues were 90–100% conserved among uncultured HIV-1 *nef* alleles derived directly from infected individuals (Shugars *et al.,* 1993). Structural analysis of Nef protein using nuclear magnetic resonance spectroscopy indicates that this cleavage site is contained in a region just N terminal to a well-folded "core" domain that contains the SH3-binding surface (Freund *et al.,* 1994a; Grzesiek *et al.,* 1996a; Lee *et al.,* 1996). These structural features have led to the suggestion that proteolytic cleavage in the virion would release the well-folded core of Nef from the amino-terminal membrane anchor, potentially freeing the Nef core for action within the virion or within the target cell (Freund *et al.,* 1994a; Pandori *et al.,* 1996).

In addition to their role as a target site for the HIV-1 protease, residues that compose the cleavage site (in particular, W57 and L58) have been shown to participate in an intramolecular interaction with a hydrophobic pocket formed by two alpha helices in the well-folded Nef core (Grzesiek *et al.,* 1996a, 1996b; Lee *et al.,* 1996). Together, residues of the cleavage site and this pocket form a hydrophobic patch on Nef protein that appears to have a weak binding affinity for a peptide sequence derived from the cytoplasmic tail of CD4 (Grzesiek *et al.,* 1996b). These structural and biochemical features have suggested that these residues may be involved in a direct binding interaction between CD4 and Nef. In support of such an interaction, certain cleavage site residues, specifically residues 57–59, have been shown to be required for CD4 down-regulation, whereas dispensable for the localization of Nef to endocytic vesicles (Greenberg *et al.,* 1997). The relationship between these observations, the intravirion proteolytic processing of Nef, and enhancement of viral infectivity is unclear.

The inclusion of Nef protein and its proteolytic processing within virions is of unknown biological relevance. Indeed, a recent mutational analysis has suggested that generation of the carboxyl-terminal core domain of Nef by intravirion proteolysis is insufficient for enhancement of infectivity (Miller *et al.,* 1997). To investigate further the relevance of these phenomena, a panel of *nef* mutants characterized by a variety of intravirion cleavage phenotypes has been generated. The infectivities of these mutant viruses have been measured after production using a variety of cell types. The data indicate that cleavage of Nef protein in virions does not correlate directly with Nef-mediated enhancement of infectivity. However, amino acid residues at the protease cleavage site are critically important for enhancement of viral infectivity. Interestingly, the importance of certain of these amino acid residues for optimal infectivity is manifest most dramatically when viruses are produced using T cells (lymphoblastoid cell lines or primary lymphoblasts derived from PBMCs) and not when viruses are produced using 293 or HeLa cells. The role of these amino acids in CD4 down-regulation and the relationship between CD4 down-regulation and enhancement of infectivity by this Nef domain are described.

RESULTS

Amino acid residues 54–60 in HIV-1 Nef govern the specificity of intravirion proteolytic processing and are necessary for optimal viral infectivity

To modulate cleavage of Nef protein within virions, NL4–3-based proviral mutants containing altered amino acid sequences at the putative cleavage site were constructed. Specificity of the HIV-1 protease is determined

TABLE 1

Amino Acid Sequences of Cleavage Site Mutants in NL4-3 Nef

Wild-type NL4-3 Nef	-SNTNAACAW[a]LEAQEEEEVGFP-
Δ54–57	-SNTNALEAQEEEEVGFP-
Δ56–60	-SNTNAACQEEEEVGFP-
Δ58–61	-SNTNAACAWEEEEVGFP-
Δ56–60 + Gag	-SNTNAACATIMMQRGQEEEEVGFP-
Δ54–57 + ATIM	-SNTNAATIMLEAQEEEEVGF-

[a] Indicates the cleavage site of recombinant Nef *in vitro* (Freund *et al.,* 1994b; Gaedigk-Nitschko *et al.,* 1995).

by contact with four amino acids on either side of the scissile bond; the predicted octameric cleavage site of NL4–3 Nef consists of amino acid residues 54–61, the peptide sequence ACAWLEAQ (Freund *et al.,* 1994b; Gaedigk-Nitschko *et al.,* 1995; Skalka, 1989). Using PCR-based mutagenesis, mutant sequences encoding the *nef* alleles were constructed (Table 1); these included three alleles in which partially overlapping in-frame deletions were engineered within the putative octameric cleavage site and two alleles in which Nef peptide sequences were replaced with sequences derived from a cleavage site within Gag. These mutant alleles were cloned into the pNL4–3 vector by exchanging the wild-type *nef* sequence between *Xho*I and *Pml*I with the *Xho*I–*Pml*I fragments containing the desired mutations. The sequences of all proviral mutants were confirmed by DNA sequencing. Proviral plasmids containing the mutated *nef* alleles as well as the wild-type pNL4–3 and the *nef*-negative pDS (Chowers *et al.,* 1994), which encodes two premature termination codons (one at codon 9 and the other at codon 21), were used to transfect 293 cells. After 72 h, virus-containing supernatants were clarified of cellular debris by centrifugation at 1000*g* followed by passage through 0.45-μm nitrocellulose filters. Virions then were purified by gel filtration chromatography using S-1000 Sephacryl columns as described previously (McKeating *et al.,* 1991; Pandori *et al.,* 1996). For each virus, fractions containing the peak concentrations of p24 antigen were combined, and equal amounts of p24 antigen were concentrated by centrifugation at 23,500*g* before analysis by immunoblot using a sheep, polyclonal, Nef-specific antiserum. As shown previously and in Fig. 1, virion-associated Nef protein was detected as both the 27-kDa full-length and 20-kDa cleaved forms.

The immunoblot shown in Fig. 1 demonstrates the variety of intravirion Nef cleavage patterns that characterized these mutants. Importantly, virion preparations of each mutant appeared to contain similar amounts of Nef protein, indicating that these mutant gene products were stably expressed to a degree such that membrane localization and subsequent packaging of Nef protein into virions could occur. These data indicated that residues 54–60 were critical for proteolytic processing of Nef within virions. Deletion of five amino acids around the putative scissile bond (Δ56–60, Fig. 1) and deletion of the four amino acids constituting the amino half of the cleavage site (Δ54–57, Fig. 1) both blocked cleavage, whereas in-frame deletion of the four amino acids constituting the carboxyl half of the cleavage site (Δ58–61, Fig. 1) did not affect cleavage substantially.

Two additional mutants were constructed and characterized, each of which contained heterologous cleavage sites in place of the native protease cleavage site within Nef (Table 1). These mutants were designed to allow cleavage of Nef using amino acid residues unrelated to the native sequence. In the case of the mutant Δ56–60+Gag, this was accomplished by in-frame substitution of the amino acid residues ATIMMQRG, which forms an efficient proteolytic cleavage site in the Gag polyprotein of HIV-1 between P2 and nucleocapsid, in place of the native Nef sequence AWLEA (Erickson-Viitanen *et al.,* 1989). A mutant was also constructed that contained the chimeric cleavage site ATIMLEAQ, which contains the amino half of the P2/NC cleavage site and the carboxyl half of the Nef cleavage site (Δ54–57+ATIM). This construct was designed to restore cleavage to the Δ54–57 mutant while potentially preserving with wild-type sequence the 20-kDa, carboxyl-terminal Nef fragment. Both the Δ56–60+Gag (Fig. 1) and the Δ54–57+ATIM (Fig. 1) viral mutants packaged and cleaved Nef with an efficiency similar to, if not greater than, that of wild-type virus. These data indicated that the proteolytic cleavage site found in native HIV-1 Nef protein is exchangeable

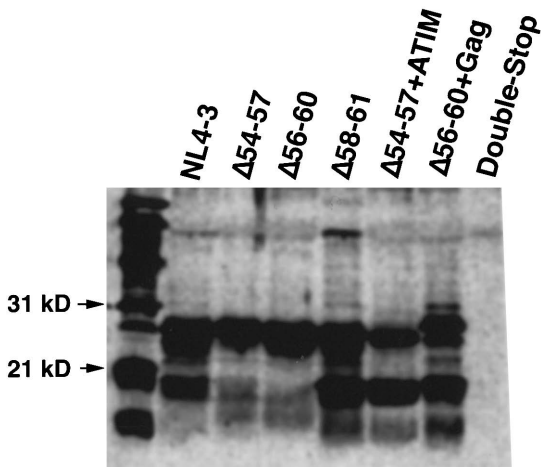

FIG. 1. Immunoblot for Nef in wild-type and *nef* mutant virions. Virions produced by transfected 293 cells were purified by filtration through 0.45-μm nitrocellulose filters followed by gel filtration chromatography using S-1000 Sephacryl columns. The purified virions were concentrated by centrifugation, and 1 μg of p24 of each mutant was applied to each lane of a 14% polyacrylamide gel. After transfer to a nitrocellulose membrane, the blot was probed with a polyclonal sheep anti-Nef antiserum followed by detection with peroxidase-conjugated donkey anti-sheep secondary antibody and development with enhanced chemiluminescent substrate. Nomenclature is as described in Table 1; "Double-stop" contains two premature termination codons in the 5′ terminus of the *nef* gene (Chowers *et al.,* 1994).

biochemically with a heterologous HIV protease cleavage site from the Gag protein. Mutant virions produced from acutely infected CEM cells revealed cleavage phenotypes similar to those of the 293 cell-derived virions shown in Fig. 1 (data not shown), indicating that cleavage of Nef protein within virions was not producer cell specific.

To determine whether proteolytic processing of Nef protein within virions correlated with enhancement of infectivity, stocks of these mutant viruses were prepared by transfection of CEM cells with proviral DNA and propagation of the resulting virus to relatively high titer. Viral stocks were also produced by allowing the cultures to become chronically infected and then harvesting the virions produced transiently during a 72-h interval. The infectivities of these viral stocks were quantified using a previously described infectious center assay, which uses the CD4 positive HeLa cell line HT4–6C as the target (Pandori *et al.,* 1996). Exposure of monolayers of these cells to virus stocks for 3 days results in the formation of readily visible syncytia after fixation of the cells and staining with crystal violet. These syncytia were counted, and the amount of p24 antigen required for a syncytium-forming unit was calculated for each viral stock. The infectivity of each viral stock was then expressed relative to the infectivity of wild-type virus.

Figure 2 shows the relative infectivities of each mutant viral stock compared with the wild-type (NL4–3) and *nef*-negative (DS or Δnef) controls. As previously described, the infectivity of *nef*-negative virus was only 10% that of wild-type virus. The infectivities of the mutants containing deletions within the cleavage site of Nef (Δ54–57, Δ56–60, and Δ58–61) were indistinguishable from that of the *nef*-negative virus and in no case exceeded 20% of wild-type levels. The infectivities of mutants containing cleavage sites derived from Gag (Δ54–57+ATIM and Δ56–60+Gag) were also indistinguishable from that of *nef*-negative virus. The relative infectivities of the mutants were identical whether virions were produced during spreading infection in cultures of transfected CEM cells (Fig. 2A) or produced transiently from chronic producer CEM cells (Fig. 2B); these data excluded the possibility of a systematic artifact introduced by the comparison of virions that had propagated at different rates. The inability of the alleles Δ58–61, Δ56–60+Gag, and Δ56–60+ATIM to enhance infectivity, despite their ability to generate Nef proteins that were incorporated and cleaved in the virion to an extent similar to that of the wild type, indicated that the cleavage of Nef in virions was not sufficient for Nef-mediated infectivity enhancement. Nevertheless, the amino acid residues that constitute the proteolytic cleavage site appeared to be essential for optimal infectivity.

Amino acid residues at the cleavage site are important for Nef-mediated CD4 down-regulation

The observations described above suggested that this domain in Nef might perform a function in addition to provision of a cleavage site that is important for enhancement of infectivity. Structural data have suggested that several of the residues of the cleavage site participate in the formation of a hydrophobic surface on Nef that appears to bind with weak affinity to a peptide sequence corresponding to the cytoplasmic tail of CD4 (Grzesiek *et al.,* 1996b). These structural data suggest that a second function performed by this domain might be related to CD4 down-regulation.

To determine the role of the cleavage site domain in CD4 down-regulation, the mutant *nef* alleles Δ54–57, Δ56–60, and Δ58–61 were cloned into the eukaryotic expression vector pCI*neo* for testing in a 293 cell-based assay. Each Nef expression vector was used together with a CD4 expression vector and a vector that expresses the green fluorescent protein (GFP) to transfect 293 cells. After 72 h, the cells were stained using the monoclonal antibody Leu3a conjugated to phycoerythrin and analyzed for surface expression of CD4 using flow cytometry. Figure 3A shows the expression of CD4 on the surface of cells transfected with the various mutant *nef* alleles compared with cells transfected with wild-type *nef* or the empty vector (non-*nef*-expressing) control. Under the conditions of this assay, expression of wild-type Nef reduced the percentage of CD4-positive cells by ~3-fold, from 72% to 25% of the GFP-positive population. In contrast, the mutants Δ54–57, Δ56–60, and Δ58–61 had little or no detectable effect on CD4 expression. Immunoblot analysis of the transfected cells used in these flow cytometry experiments confirmed equivalent expression of all Nef gene products (Fig. 3B). These data indicated that residues of the proteolytic cleavage site were important for CD4 down-regulation as well as for optimal infectivity.

Interestingly, the electrophoretic mobility of the Δ54–57 Nef protein appeared to be retarded relative to wild-type Nef and the other deletion mutants. Similar anomalous mobility was observed for an alanine substitution mutant, W57A/L58A (data not shown). Such mobility shifts were not observed for the Δ58–61 or Δ56–60 mutants. Curiously, the anomalous mobility of Δ54–57 Nef was not observed in immunoblots of virions produced by 293 or CEM cells (Fig. 1; data not shown).

The infectivity phenotypes of the cleavage site mutants depend on the producer cell type

To examine the possibility of a causal relationship between the effect of the cleavage site domain on CD4 down-regulation and its effect on viral infectivity, the infectivities of the cleavage site mutants were measured after virion production by CD4-negative cells. Mutant

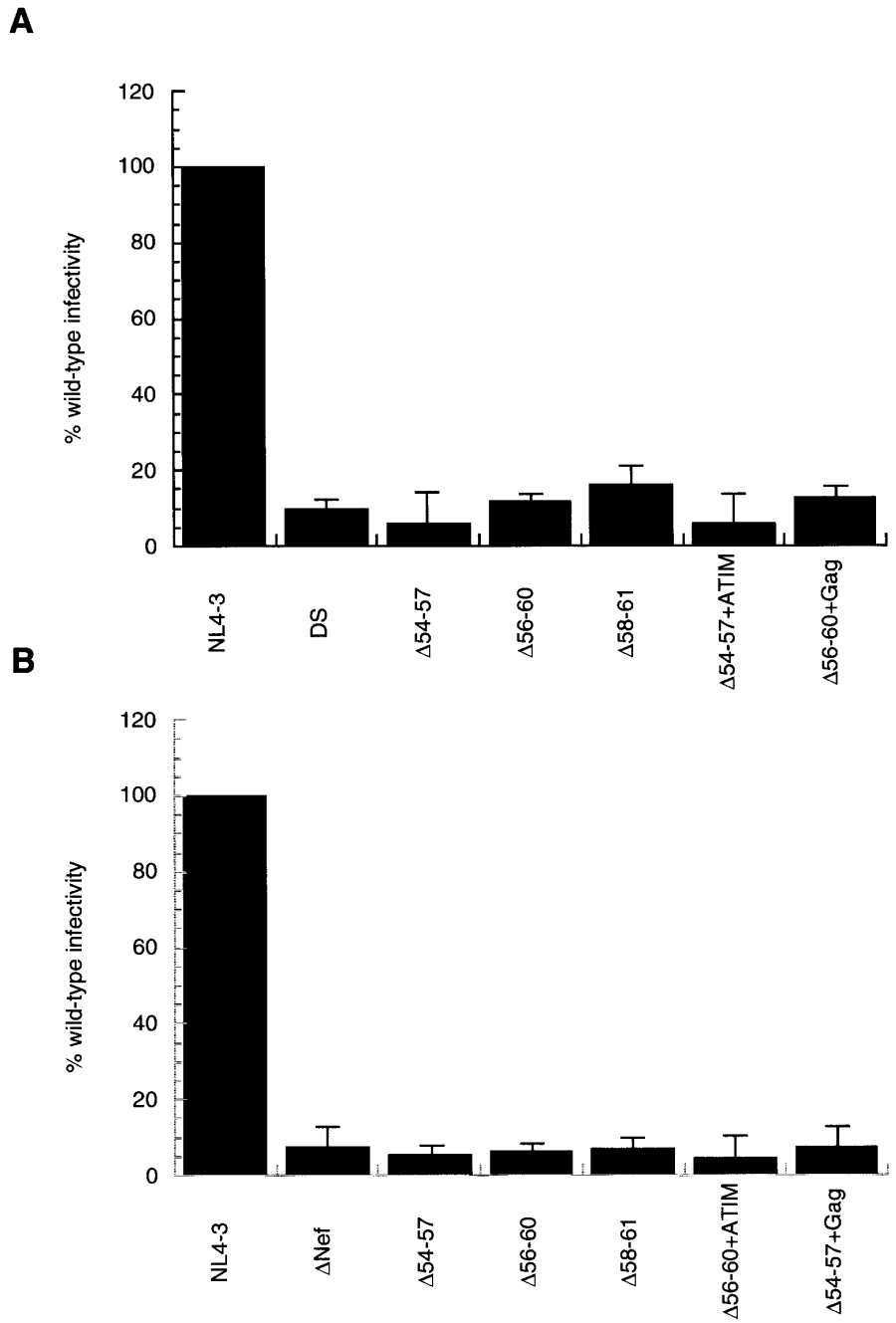

FIG. 2. Infectivity of nef-mutant viruses produced from CEM cells. (A) Viral stocks produced during spreading infection after transfection of CEM cells with the indicated proviral clones. (B) Viral stocks produced by transient production from chronic producer CEM cells. Virus-containing supernatants were quantified by p24 ELISA. The viral stocks were used to inoculate monolayers of HeLa-CD4 (HT4–6C) cells in a syncytium formation assay, and the percent wild-type infectivity was calculated as described in the Materials and Methods. The values in A are the average of duplicate assays of each viral stock; error bars are the standard deviation of the mean. The results are representative of three independently produced sets of viral stocks. The values in B are the average of two independently produced sets of viral stocks, each titered twice; error bars are the standard deviation of the mean. Nomenclature is as described in Table 1 "DS" contains two premature termination codons in the 5′ terminus of the *nef* gene (Chowers *et al.,* 1994). "Δnef" contains a deletion in the 5′ terminus of the *nef* gene, which introduces a frameshift (Spina *et al.,* 1994).

viruses were prepared by transient transfection of 293 cells, a CD4-negative, adenovirus DNA-transformed human embryonic kidney cell line. The infectivities of the virus stocks were measured using the syncytium formation assay described above (Fig. 4). The infectivity of the *nef*-negative virus was 10% that of the wild type, similar to the data obtained for the CEM-derived viruses shown in Fig. 2. However, certain cleavage site mutants were minimally if at all impaired after production using 293 cells. Specifically, the mutants Δ54–57 and Δ54–57+ATIM were nearly as infectious as the wild type (80–70% of wild-type infectivity) when produced using

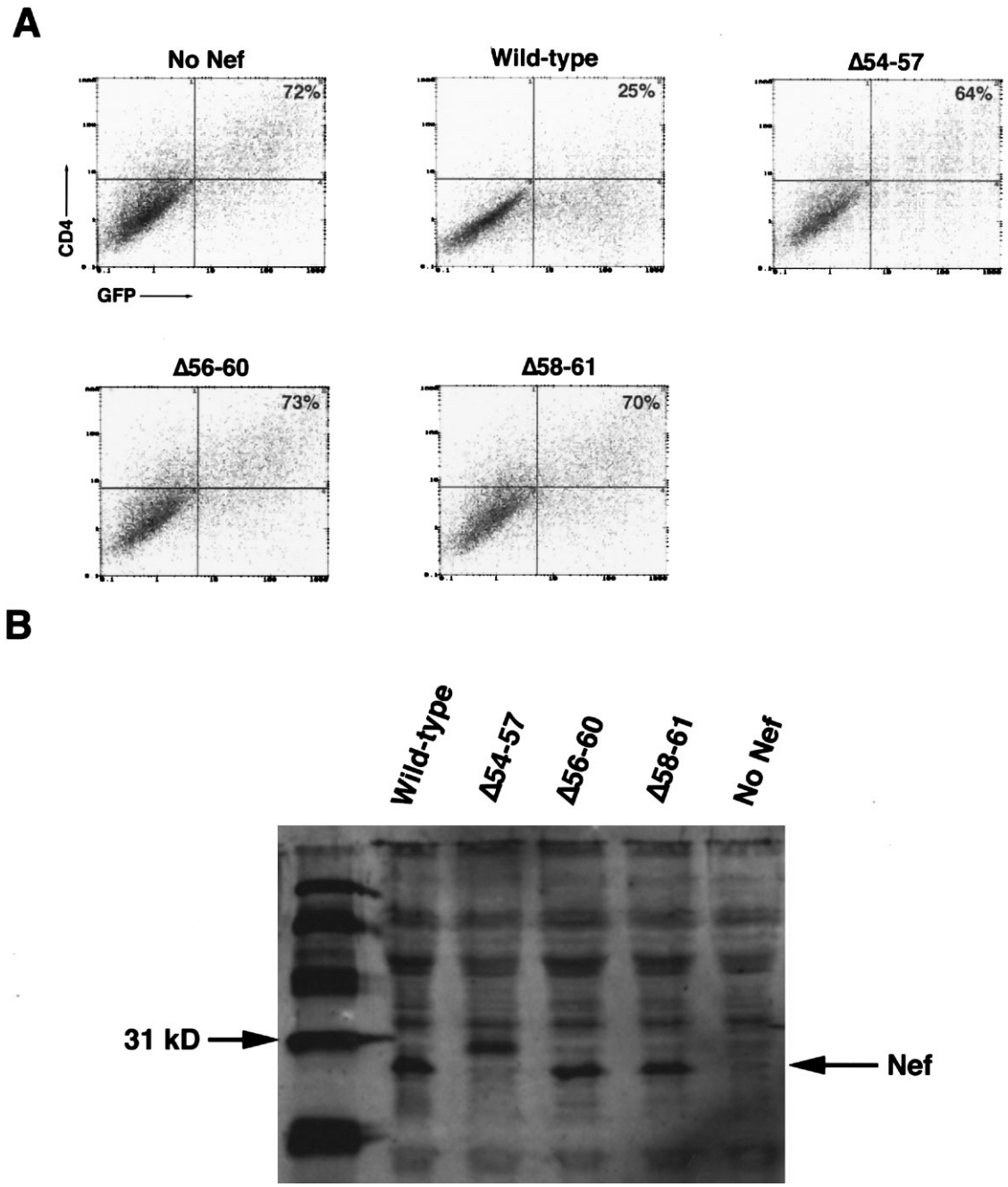

FIG. 3. (A) CD4 down-regulation in transfected 293 cells. Cells were transfected with 1 μg of a GFP expression vector, 1 μg of a CD4 expression vector (pCMX-CD4) and either pCIneo or a pCI*neo*-based *nef* expression vector (2 μg) as described in Materials and Methods. After 72 h, cells were stained with anti-CD4 (Leu 3a) conjugated to PE and analyzed by two-color flow cytometry for GFP (the transfection marker) and PE (surface CD4). The gates for GFP-positive and PE- (CD4)-positive cells were set as described in the Materials and Methods. The percentage shown in each analysis is the percentage of GFP-positive cells that were also positive for PE (CD4). (B) Immunoblot of transfected 293 cells used in the CD4 down-regulation assay. The transfected 293 cells (10^5) used in the CD4 down-regulation assay shown in Fig. 3A were assayed by immunoblot for Nef protein as described in the Materials and Methods.

293 cells (Fig. 4); in contrast, these mutants were phenotypically *nef* defective (10% of wild-type infectivity) when produced using CD4-positive, T lymphoblastoid CEM cells (Fig. 2). The Δ58–61 mutant was characterized by an intermediate phenotype when produced using 293 cells (50% of wild-type infectivity), in contrast to the *nef*-defective phenotype observed when produced using CEM cells. The mutant Δ56–60 was characterized by a relatively defective infectivity phenotype whether 293 or CEM cells were used as viral producers.

These data indicated that the viral determinants of Nef-mediated infectivity enhancement varied in different producer cell environments. The greatest variation was revealed by the mutant Δ54–57; residues 54–57, although

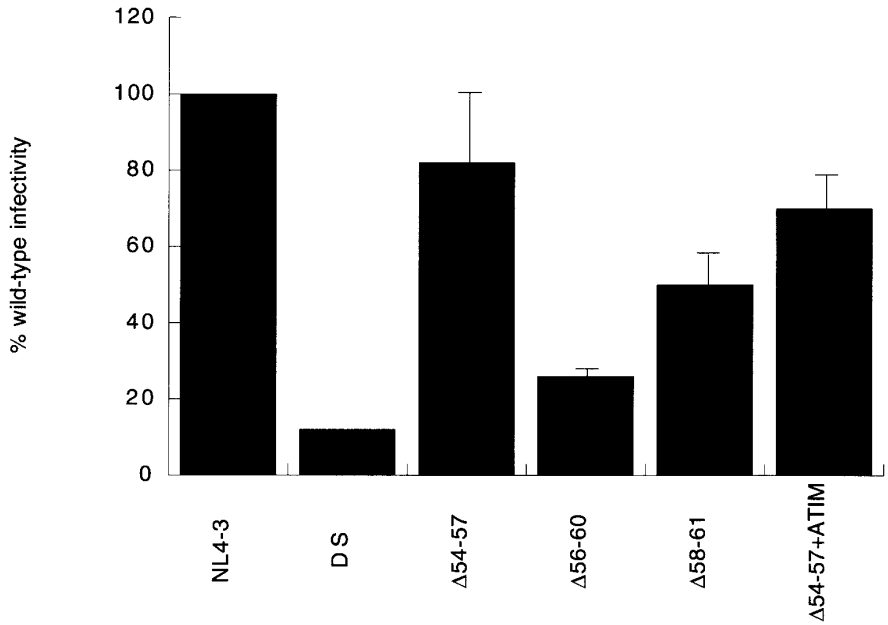

FIG. 4. Infectivity of *nef* mutant viruses produced from 293 cells. Virus-containing supernatants were harvested from transfected 293 cells and quantitated by p24 ELISA. The viral stocks were used to inoculate monolayers of HeLa-CD4 (HT4–6C) cells in a syncytium formation assay, and the percent wild-type infectivity was calculated as described in the Materials and Methods. The values are the average infectivities of two independently produced viral stocks, each titered twice; error bars are the standard deviations of the mean.

required in CEM T cells, were essentially dispensable in 293 cells. Because Δ54–57 Nef failed to cleave in virions (Fig. 1), these data indicated that when virions were assembled in 293 cells, intravirion cleavage was unnecessary for Nef-mediated infectivity enhancement. Under these producer cell conditions, deletion of residues 54–57 further appeared to define a novel class of *nef* allele that is specifically defective in CD4 down-regulation but able to enhance viral infectivity.

Given the cell type-specific infectivity defect of the Δ54–57 mutant, we considered further the possibility that CD4 down-regulation might be causally linked to at least a fraction of the net effect of Nef on viral infectivity when virions are produced from CD4-positive cells. First, we performed experiments in which viruses were produced from 293 cells that had been transfected with the proviral plasmids either with or without cotransfection of a CD4 expression plasmid. Overexpression of CD4 in this setting reduced the infectivities of both wild-type and *nef*-negative viruses to unmeasurable levels (data not shown). These results were qualitatively similar to previous studies indicating an inhibitory effect of CD4 overexpression in the producer cell on viral infectivity (Marshall *et al.,* 1992). However, we considered these experiments to be potentially problematic with respect to the relevance of the levels of CD4 expression achieved; artifacts resulting from supraphysiological levels of CD4 expression, for example Env shedding or sequestration of Env in the endoplasmic reticulum, might account for the observed inhibition of infectivity.

To provide a level of CD4 that was as relevant as possible as the test variable, we identified producer cell lines that were similar in origin but differed with respect to the presence or absence of physiological levels of CD4 expression. To identify these lines, the levels of CD4 expression on the surfaces of two CD4-positive HeLa cell lines [HeLa-HT4–6C and HeLa-1022 (Chesebro *et al.,* 1991)] were compared using flow cytometry with those of CEM cells and primary CD4-positive T lymphoblasts (data not shown). The surface expression of CD4 on cells of the HeLa-1022 line was quantitatively most similar to that of CEM cells and primary lymphoblasts (Fig. 5). Cells of this CD4-positive HeLa cell line were then compared with CD4-negative HeLa cells as viral producers. By comparison to the data obtained using CD4-negative HeLa cells, CD4 expression on the surface of HeLa-1022 cells had no effect on the infectivity of wild-type virus but increased modestly (2-fold) the infectivity of *nef*-negative virus (Fig. 6). As was the case when produced using 293 cells, the infectivity of the Δ54–57 mutant was relatively unimpaired when produced using CD4-negative HeLa cells, retaining $>$70% of wild-type activity (Fig. 6). When produced using the CD4-positive HeLa-1022 cells, the Δ54–57 mutant similarly retained $\sim$60% of wild-type infectivity. Also in concordance with the data obtained using 293 cells as producers, the Δ58–61 mutant retained 70% of wild-type infectivity when produced using CD4-negative HeLa cells. Like the Δ54–57 mutant, the infectivities of Δ58–61 viruses produced by CD4-negative HeLa and CD4-positive HeLa-1022 cells were not significantly different. Unexpectedly, but similar to the effects observed for the *nef*-negative control, the infectivity of

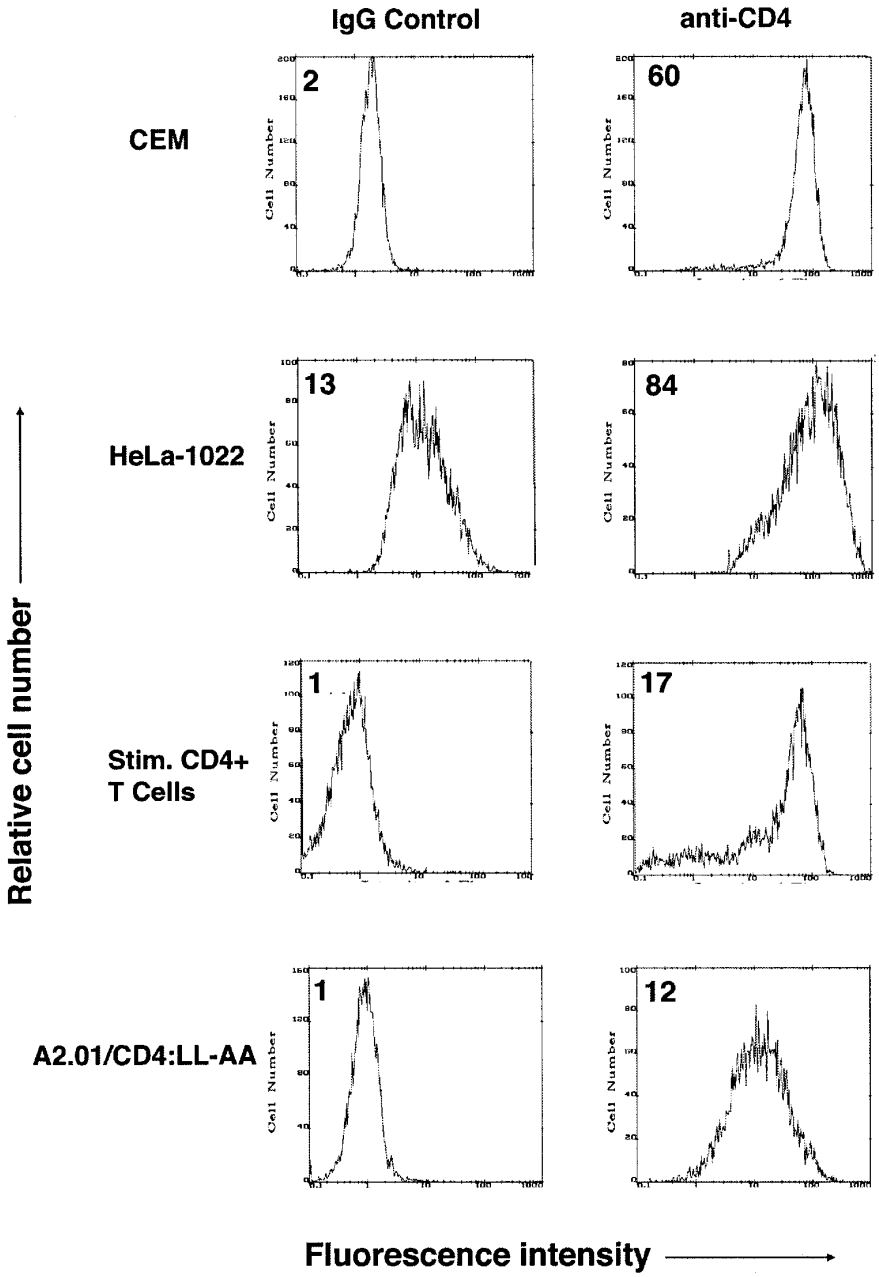

FIG. 5. Surface CD4 expression on CEM cells, HeLa-1022 cells, A2.01/CD4: LL-AA cells, and purified CD4-positive primary T cells. Cells (2×10^5) of each type were stained with anti-CD4 (Leu3a) conjugated to phycoerythrin and analyzed by flow cytometry. The value shown in each histogram is the mean peak fluorescence for PE staining. CD4-positive primary T cells were isolated from PBMC as described previously (Pandori *et al.,* 1996). The primary cells were stimulated with PHA (3 μg/ml) and interleukin-2 (5 U/ml) 2 days before analysis by flow cytometry.

the Δ56–60 mutant was 2-fold greater (67% of wild-type infectivity) when produced from CD4-positive HeLa-1022 cells than when produced from CD4-negative HeLa cells (34% of wild-type infectivity). Although these data suggested that CD4 expression may modulate slightly the phenotype of certain *nef* mutants, they also indicated that CD4 expression alone was unlikely to account for the cell type-specific contribution of cleavage site residues to Nef-mediated infectivity enhancement.

It remained possible, however, that CD4 played a significant role in the virological function of the cleavage site domain in cooperation with another T cell-specific factor. To examine this possibility, viral stocks of the mutants Δ54–57, Δ56–60, and Δ58–61 were prepared by transfection and subsequent propagation of virus using A2.01/CD4:LL-AA cells (Moutouh *et al.,* 1998). This cell line was obtained by transduction of the CD4-negative T cell clone A2.01 with a retroviral vector containing a CD4 gene encoding alanine substitutions of leucine residues 413 and 414 in the CD4 cytoplasmic tail, followed by selection of cells expressing high levels of CD4 from the bulk population using flow cytometry (Moutouh *et al.,*

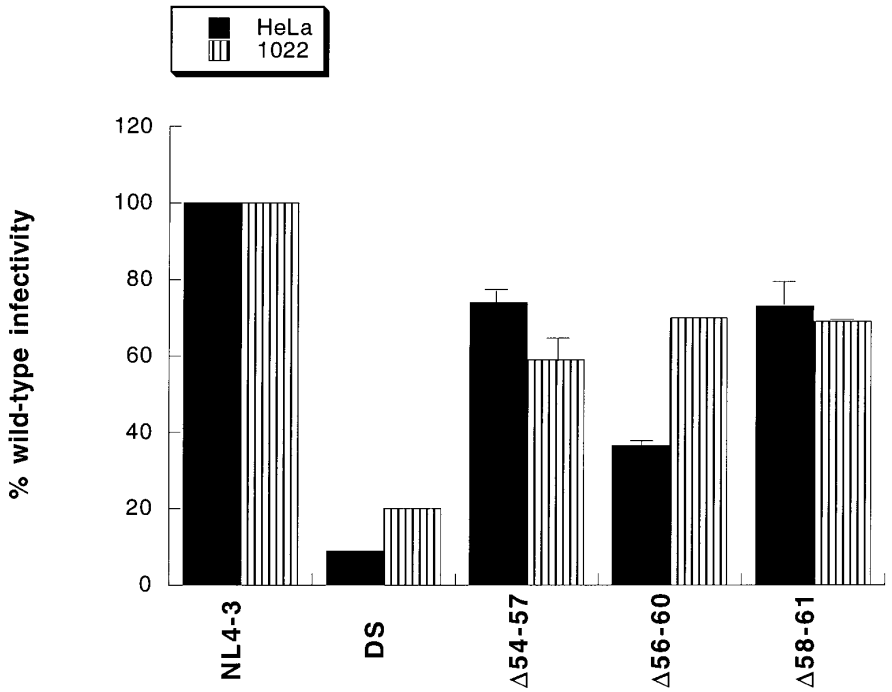

FIG. 6. Infectivity of *nef* mutant viruses produced from either CD4-negative HeLa cells or CD4-positive HeLa-1022 cells. Virus-containing supernatants were harvested from transfected cells and quantified by p24 ELISA. The viral stocks were used to inoculate monolayers of HeLa-CD4 (HT4–6C) cells in a syncytium formation assay, and the percent wild-type infectivity was calculated as described in the Materials and Methods. The values are the averages of duplicates; error bars are the standard deviation of the mean. The results are representative of two independently produced sets of viral stocks. The absolute infectivities for viruses produced from CD4 negative HeLa cells (in pg of p24 per syncytium-forming- unit) were NL4–3, 1.0; DS, 11.2; Δ54–57, 1.4; Δ56–60, 2.8; and Δ58–61, 1.4. The absolute infectivities for viruses produced from CD4-positive HeLa cells (in pg of p24 per syncytium-forming unit) were NL4–3, 1.0; DS, 5.1; Δ54–57, 1.8; Δ56–60, 1.5; Δ58–61, 1.5.

1998). The leucine residues 413 and 414 have been reported as essential for binding of the cytoplasmic tail of CD4 to Nef, and alanine substitution of these residues renders the CD4 molecule refractory to Nef-mediated CD4 down-regulation (Aiken *et al.,* 1994; Grzesiek *et al.,* 1996b). The infectivities of the cleavage site mutants

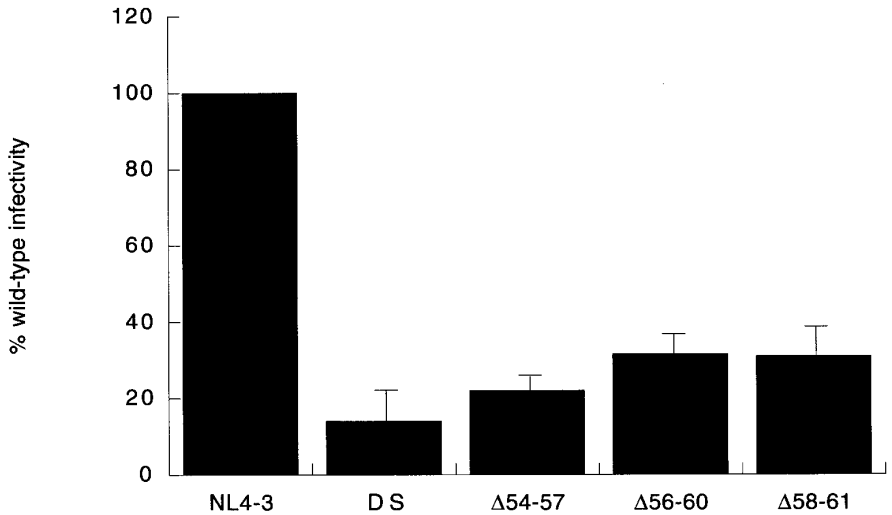

FIG. 7. Infectivity of *nef* mutant viruses produced using A2.01/CD4:LL-AA cells, which express a mutant CD4 molecule (LL-413, 414-AA mutation of the cytoplasmic tail). Virus-containing supernatants were harvested from transfected cells and quantified by p24 ELISA. The viral stocks were used to inoculate monolayers of HeLa-CD4 (HT4–6C) cells in a syncytium formation assay, and the percent wild-type infectivity was calculated as described in the Materials and Methods. The values are the average of duplicate assays of each viral stock; error bars are the standard deviation of the mean. The results are representative of two independently produced sets of viral stocks.

produced using these cells are shown in Fig. 7. As has been the case for all producer cells studied, the infectivity of *nef*-negative virus was markedly less than that of the wild type (15% of wild-type infectivity). Similar to the results obtained using CEM cells as viral producers, and unlike the results obtained using 293 and HeLa cells, all three cleavage site mutants (Δ54–57, Δ56–60, and Δ58–61) were markedly impaired in infectivity when produced using A2.01/CD4:LL-AA cells. However, the magnitude of the defects of these mutants when produced from A2.01/CD4:LL-AA cells was slightly less than when produced from CEM cells. This observation suggested that CD4 down-regulation might contribute a small fraction of the net *nef* phenotype when virions are produced from CD4-positive T cells. Nevertheless, these data indicated that the cleavage site residues remained critical for infectivity enhancement in the T cell environment even when the potential for an interaction between Nef and CD4 has been eliminated and CD4 down-regulation by Nef was predicted not to occur. Together, the data of Figs. 6 and 7 argue against a hypothesis in which cleavage site residues are important for infectivity solely because they are required for CD4 down-regulation.

Cleavage site residues are essential for infectivity enhancement when virions are assembled in primary lymphoblasts

The above data indicated that residues of the cleavage site performed a function essential to infectivity enhancement when virions were produced using continuous T cell lines. To establish the relevance of these observations, the infectivities of the cleavage site mutants were evaluated after production using primary PBMCs. High-titer viral stocks produced from CEM cells were used to infect PBMCs that were stimulated with PHA and interleukin-2. Twelve days after the initiation of infection, virus-containing supernatants were harvested, and viral infectivities were measured using the syncytium formation assay described above (Fig. 8A). In concordance with the data obtained using CEM and A2.01 T cells as viral producers, the infectivities of the cleavage site mutants were indistinguishable from that of *nef*-negative virus. These data underscore the importance and relevance of this domain with respect to Nef-mediated infectivity enhancement. They also support the generalization that the molecular action of this domain is most critical within the T cell environment.

To establish further the relevance of this domain, the rates of propagation of the cleavage site mutants were determined in primary lymphoblasts (Fig. 8B). The mutants Δ56–60 and Δ58–61 were intermediate in growth rate, whereas the mutant Δ54–57 was characterized by a growth rate that was almost as attenuated as that of *nef*-negative virus. These data indicated that the virological importance of this domain was not limited to the context of HeLa-CD4 cells as the targets for infection.

DISCUSSION

The protease cleavage site in HIV-1 Nef appears to be a focal point within the protein for a variety of biochemical, structural, and functional effects. First identified using cell-free reactions between recombinant proteins, the cleavage site acquired biological relevance with the observation that Nef protein is incorporated into the virion and is processed there by the viral protease (Freund *et al.,* 1994b; Gaedigk-Nitschko *et al.,* 1995; Pandori *et al.,* 1996; Welker *et al.,* 1996). These findings suggested a role for virion incorporation and proteolytic cleavage in Nef-mediated enhancement of virion infectivity. Structural data have indicated that the cleavage site is located between a relatively unstructured amino-terminal region and a relatively well-folded, globular core region in Nef that contains potential protein-interaction domains such as an SH3-binding surface (Grzesiek *et al.,*1996a; Lee *et al.,* 1996). In addition, residues of the cleavage site, in particular W57 and L58, participate in an intramolecular interaction with residues of a hydrophobic pocket formed by two alpha helices of the core (Grzesiek *et al.,* 1996a). Together, residues of the cleavage site and this pocket form a hydrophobic surface with a weak binding affinity for a peptide sequence within the cytoplasmic tail of CD4 (Grzesiek *et al.,* 1996b). These findings have suggested a direct role for these residues in Nef-mediated CD4 down-regulation. Here, we have sought to define the relationships between these biochemical and structural features and the functional properties of Nef with respect to CD4 down-regulation and infectivity enhancement. In summary, residues 54–61 are the primary target within Nef for the viral protease during virion maturation, are essential for CD4 down-regulation, and are critical for enhancement of infectivity in a conditional fashion that depends on the producer cell environment.

When virions are assembled in T cells, residues of the cleavage site are essential for optimal infectivity; cleavage itself, however, is insufficient for the virological effect of this domain. Mutational analysis of residues 54–61 of HIV-1 Nef confirmed that the cleavage site used within virions is closely related if not identical to that observed using recombinant proteins. The infectivities of viruses that contain cleavage-resistant Nef proteins (Δ54–57 and Δ56–60) were equivalent to that of *nef*-defective viruses. However, mutants of this region for which virion-associated Nef was cleaved efficiently were nevertheless defective for enhancement of infectivity. These included a mutant in which the carboxyl half of the cleavage site was deleted (Δ58–61) and two mutants in which residues essential for cleavage were replaced with residues of the Gag P2/NC cleavage site. These data did not support

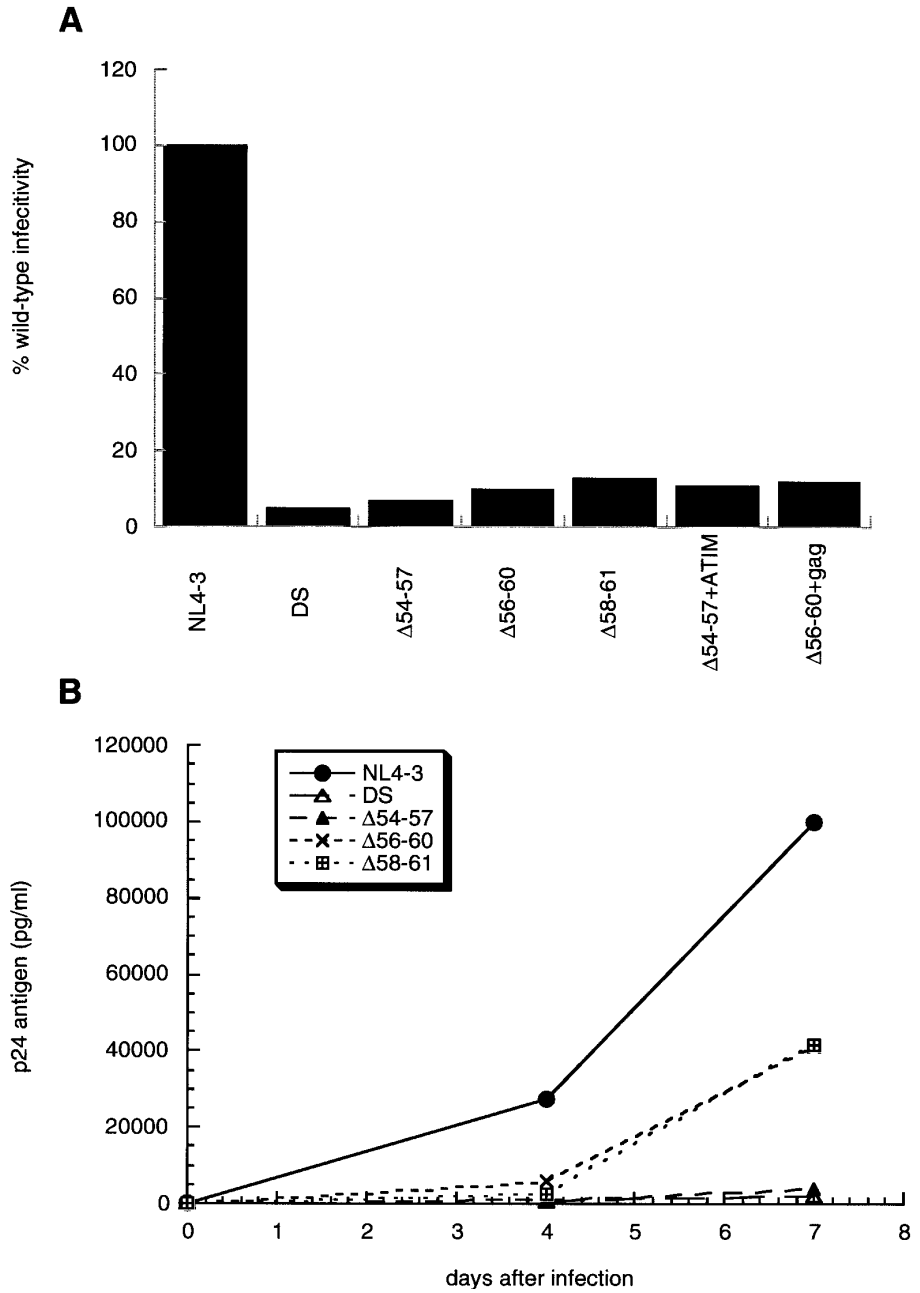

FIG. 8. (A) Infectivity of *nef* mutant viruses produced from primary PBMCs. Viral stocks produced by PBMCs as described in Materials and Methods were used to inoculate monolayers of HeLa-CD4 (HT4–6C) cells in a syncytium formation assay, and the percent wild-type infectivity was calculated. The values were derived from a single titration of viral stocks. (B) Growth rates of *nef*-mutant viruses in PBMCs. Cells (2×10^6) were stimulated with PHA (3 μg/ml) and interleukin-2 (20 U/ml) 3 days before inoculation with viral stocks derived from transfected CEM cells (200 ng of p24 antigen). The inoculated cells were washed and incubated at 37°C, and the concentrations of p24 antigen in the culture supernatants were determined by ELISA.

a model in which infectivity enhancement requires proteolytic release of the well-folded Nef core for function within the virion or the target cell. Instead, these data indicated that these residues perform a function in addition to provision of a cleavage site that is required for optimal infectivity.

Certain cleavage site residues, in particular, residues 54–57, were most important to infectivity during virion assembly in CEM cells, A2.01 cells, and primary lymphoblasts and were less important during virion assembly in 293 cells and HeLa cells; these observations suggested that the hypothetical second function of this region might involve a T cell-specific molecule or process. In initial support of this hypothesis, residues of the cleavage site were found to be required for Nef-mediated CD4 down-regulation. This observation suggested that a Nef–CD4 interaction or CD4 down-regulation itself might be central to the mechanism by which this domain contributes

to infectivity. In one such scenario, down-regulation of CD4 would be an essential component of infectivity enhancement when virions are produced from CD4-positive cells but would be dispensable when virions are produced from CD4-negative cells. However, comparison of virions assembled in different producer cells suggested that CD4 down-regulation is unlikely to be the sole function of this domain. The expression of CD4 on HeLa cells in amounts comparable to that of CEM cells and primary T lymphoblasts did not create a producer cell environment in which residues 54–57 acquired a greater role in enhancement of infectivity. Furthermore, these residues were important for optimal infectivity, even during virion assembly in T cells (A2.01/CD4:LL-AA) that expressed a CD4 molecule that cannot be down-regulated by Nef.

Taken together, these data indicated that the importance of residues at the protease cleavage site for the virological function of Nef is independent of both intravirion Nef processing and cell surface CD4 down-regulation. What, then, might be the function of this domain? Notably, the characteristics of each of the three in-frame deletion mutants analyzed were unique. This observation suggests that mutations in different regions of the cleavage site affect differentially an unidentified parameter that influences the function of Nef. One possibility is that the hydrophobic surface in which certain cleavage site residues (W57 and L58) reside interacts with a cellular factor (in addition to CD4) through which Nef operates to enhance infectivity. A second possibility is that these mutations affect the formation of an intramolecular fold in Nef between residues W57 of the cleavage site and L97 of the Nef core (Grzesiek *et al.,* 1996b). Potentially, the dissimilar electrophoretic mobilities of these mutants may provide evidence for differental effects on this tertiary fold.

The explanation for the influence of the producer cell type on the infectivity phenotypes of these mutants remains elusive. It is interesting to note that differences in the infectivity phenotypes observed for cleavage site mutants of HIV-1 Nef after production by 293 and CEM cells have been described recently (Chen *et al.,* 1998). In particular, alanine substitution of residues 57 and 58 yielded a noncleavable Nef protein, and virus encoding this mutation retained significant infectivity when produced from 293 cells despite a marked defect in infectivity when produced from CEM cells (Chen *et al.,* 1998). This characterization is similar to that of the Δ54–57 mutant described herein. As mentioned above, one possibility for the cell type specificity of these mutants is that enhancement of infectivity requires an interaction between certain cleavage site residues and a cellular factor found specifically within T cells. A second possibility is that Nef performs more than one function, each of which contributes to infectivity; these functions would have different genetic determinants as well as different relative importances in various producer cells. A third possibility is that mutations at the cleavage site may disrupt the tertiary structure of Nef. Such a folding disturbance would be predicted to affect CD4 down-regulation due to the involvement of the intramolecular interaction between cleavage site residues and the Nef core in the formation of a CD4 binding surface. However, such a folding disturbance could also affect Nef-mediated infectivity enhancement, even if the cleavage site residues were not involved directly in the molecular interaction by which Nef enhances infectivity. If such misfolding caused only a partial loss of Nef function, then this loss might be manifest more profoundly in certain cell types due to different levels of Nef expression by the provirus.

Despite these uncertainties, the use of different producer cells allowed the observation of a novel genetic separation of CD4 down-regulation from enhancement of infectivity. When viruses were produced from 293 cells, the mutant Δ54–57 defined a region of Nef that is required for CD4 down-regulation but is not required for infectivity enhancement; such a Nef mutant has not been described previously. Interestingly, a mutant of the SH3-binding surface of Nef has been characterized previously by directly opposite phenotypes; this mutant is reportedly deficient in infectivity while wild type with respect to CD4 down-regulation (Goldsmith *et al.,* 1995; Wiskerchen and Cheng-Mayer, 1996).

These observations also suggest the need for caution with respect to the choice of the producer cell used for the analysis of *nef* mutants. For example, exclusive use of 293 cells as producers would have failed to characterize the Δ54–57 mutant as defective, despite its marked attenuation in cultures of primary lymphoblasts. Although the characterization of the Δ54–57 mutant using 293 cells indicated that the amino-terminal half of the cleavage site, the ability to down-regulate CD4, and the intravirion cleavage of Nef are all largely dispensable for infectivity enhancement under certain conditions of viral assembly, these observations diminish neither the critical importance of this domain for Nef function during viral assembly in T cells nor its importance for optimal replication of HIV-1 in primary T lymphoblasts.

MATERIALS AND METHODS

Plasmid construction

For construction of proviral mutants, PCR mutagenesis was performed to create in-frame deletions or amino acid substitutions in the *nef* reading frame. PCR products and the proviral vector pNL4–3 were cut with *Xho*I and *Pml*I. The cut PCR products were ligated into the pNL4–3 backbone using T4 DNA ligase (Boehringer-Mannheim) and the ligation products were used to transform *Escherichia coli*. Minipreparations of plasmid DNA from transformants were analyzed by nucleotide sequencing using *nef*-specific primers, PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit, and an ABI 373A Au-

tomated Sequencer. For construction of subgenomic *nef* expression vectors, mutant *nef* genes created by PCR mutagenesis were engineered to contain *Sal*I and *Eco*RI ends. The PCR products and the eukaryotic expression vector pCi*neo* (Promega) were cut with *Sal*I and *Eco*RI. The PCR products were ligated into the pCi*neo* vector using T4 DNA ligase followed by transformation of *Escherichia coli* with the ligation products. Transformants were screened for the presence of inserts by restriction enzyme digestion with *Xho*I and *Pml* I. Candidate vectors were sequenced as described above.

Cell lines

CEM cells were maintained in RPMI 1640 culture medium supplemented with 10% FBS, 2 mM glutamine, and penicillin–streptomycin. The 293 cells were maintained in EMEM supplemented with 10% FBS, 2 mM glutamine, and penicillin–streptomycin. HeLa cells were maintained in DMEM, which was also supplemented as above. A2.01 CD4:(LL-AA) cells were derived by transduction of CD4-negative A2.01 cells with a retroviral vector encoding a human CD4 gene in which the leucine residues at positions 413 and 414 were replaced with alanines; the cells were selected for relatively high levels of cell surface CD4 expression using flow cytometry and were maintained in supplemented RPMI 1640 medium as described with the addition of G418 to a concentration of 500 μg/ml (Moutouh *et al.,* 1998). HeLa-1022 cells and HeLa HT4–6C cells were maintained as described for the parental HeLa cells with the addition of 500 μg/ml G418 (Chesebro *et al.,* 1991). Primary cultures of PBMCs were maintained in RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, and penicillin–streptomycin. Primary cells were stimulated with PHA (to a final concentration of 3 μg/ml) and interleukin-2 (to a final concentration of 20 U/ml) 3 days before infection.

Production of viral stocks

To produce virus from CEM cells, proviral plasmid DNA (1 μg) of each virus was combined with 5 μg of Lipofectin (GIBCO BRL) and incubated at 37°C with 10^6 CEM cells in 1 ml of Optimem (GIBCO BRL) for 5 h. Transfected cells were then diluted with 3 ml of supplemented RPMI medium. Cultures were split 1:3 every 3–4 days. After 9 days, when the cytopathic effect was extensive, the cultures were centrifuged at 1000*g* to pellet the cells, and the virus-containing supernatant was passed through a 0.45-μm filter. Aliquots of filtered supernatant were frozen at −80°C. In addition, the transfected cultures of CEM cells were maintained for an additional 2–3 weeks to allow the outgrowth of chronic producer cells. These cells were washed, resuspended in fresh media, and incubated for 72 h before the collection and storage of virus-containing supernatants as described above.

To produce virus from 293 cells, 10^6 cells were plated in each 35-mm well of a six-well plate 24 h before transfection. Transfection was performed by the calcium phosphate method using the Cell-Phect transfection kit (Pharmacia) as specified by the manufacturer and with 3 μg of proviral vector. After 72 h, virus-containing supernatants were pooled, and cellular debris was removed by centrifugation at 1000*g* for 10 min. Supernatants were then passed through a 0.45-μm filter, and aliquots were frozen at −80°C.

To produce virus from HeLa or HeLa-1022 cells, 5×10^5 cells were plated in each 35-mm well of a six-well plate 24 h before transfection. Transfection was performed using Lipofectamine (GIBCO BRL). Briefly, 1 μg of proviral DNA was combined with 12 μg of Lipofectamine in 500 μl of Optimem medium, and the mixtures were laid over plated cells and incubated at 37°C for 5 h. After incubation, 15 ml of supplemented DMEM was added to the cells. After 72 h, virus-containing supernatants were pooled and centrifuged at 1000*g* for 10 min and aliquots were frozen at −80° C.

To produce virus from primary PBMC, cells (2.5×10^6) were stimulated with PHA (3 μg/ml) and interleukin-2 (20 U/ml) before incubation for 6 h at 37°C with 2 ml of viral stocks prepared by transfection of CEM cells (1×10^6 pg of p24/ml). After incubation, cells were washed three times with supplemented RPMI 1640 followed by resuspension of cells in 3 ml of medium supplemented with interleukin-2 (to a final concentration of 20 U/ml). Fresh, PHA- and interleukin-2-stimulated PBMCs (2×10^6) were added to the cultures every 4 days. After 12 days, infected cultures were centrifuged at 1000*g* for 10 min to remove cellular debris. Virus-containing supernatants were aliquoted and frozen at −80°C.

Purification of virions

For the analysis of virion-associated Nef protein, HIV-1 virions were purified essentially as described previously (Pandori *et al.,* 1996). Supernatants from cultures of 293 cells transfected with a proviral vector (pNL4–3 or a mutant vector) were pooled 72 h after transfection. Virus-containing supernatants (12 ml containing $\sim 1.2 \times 10^7$ pg of p24) were centrifuged at 800*g*, and the resulting supernatants were passed through 0.45-μm filters. The filtrates were concentrated by centrifugation at 23,500*g* for 1 h at 4°C. The virion pellets were resuspended in 100 μl of supplemented RPMI 1640 medium. The concentrated virion solutions were loaded onto 2.5-ml gel filtration columns containing Sephacryl S-1000 (Pharmacia). PBS (2 ml) was added to the column, and the eluate was collected in 100-μl fractions. Fractions were analyzed by ELISA for their content of p24. Peak fractions were pooled, quantified by p24 ELISA, centrifuged at 23,500*g* for 1 h at 4°C, and resuspended in 10 μl of Western loading buffer [63 mM Tris–HCl, pH 6.8, 12.5%

(vol/vol) glycerol, 2% (wt/vol) SDS, 5% (vol/vol) β-mercaptoethanol, and 0.003% (wt/vol) bromophenol blue].

Infectivity assay

The infectivities of virus stocks were determined using a CD4-expressing HeLa cell line (HT4–6C) in a syncytium formation assay as described previously (Pandori *et al.,* 1996). Briefly, 5-fold dilutions of each virus stock in DMEM supplemented with 4% FBS, 2 mM glutamine, 0.5 μg/ml Polybrene, and 8 μg/ml DEAE–dextran were placed on a monolayer of HT4–6C cells in microtiter wells. Three days later, the wells were fixed in methanol and stained with crystal violet, and the syncytia were counted. Absolute infectivities of each viral stock were calculated by determining the number of syncytium-forming units per picogram of p24 of virus. The percent wild-type infectivity of each mutant was calculated by dividing the infectivity of the mutant by the infectivity of the wild-type virus and multiplying by 100.

CD4 down-regulation assay

For determination of CD4 down-regulation by mutant nef alleles, 293 cells were cotransfected with phGFP-S65T (Clontech) (1 μg), pCMX-CD4 (1 μg) (Aiken *et al.,* 1994), and either pCineo (2 μg) or a pCi*neo* vector engineered to express either wild-type *nef* or a mutant *nef* gene (2 μg) by the calcium phosphate method. To set the gate for GFP-positive cells, cells transfected with pCMX-CD4 only were used. To set the gate for CD4-positive cells, cells transfected with phGFP-S65T only were used. After 72 h, transfected cells ($\sim 2 \times 10^6$) were harvested and centrifuged at 800*g* for 5 min. Cells were resuspended in 200 μl of PBS (pH 7.4), 2% FBS, and 0.1% sodium azide. Then, 100 μl of cells were saved for Western blot analysis. To the remaining 100 μl of cells, 10 μl of Leu3a anti-CD4 (Becton-Dickenson) conjugated to phycoerythrin (PE) was added, and the cells were stained for 30 min at 4°C. Cells then were washed twice in 2 ml of PBS supplemented as described above. Washed cells were resuspended in 500 μl of PBS without FBS, and 500 μl of 2% paraformaldehyde was added as a fixative. Fixed cells were analyzed for GFP expression and PE staining by two-color flow cytometry.

Immunoblot assays

Immunodetection of Nef protein was performed essentially as described previously (Pandori *et al.,* 1996). Samples were resolved in a 4% stacking, 15% resolving polyacrylamide minigel with the Laemmli discontinuous buffer system and electroblotted onto 0.2-μm pore size nitrocellulose membranes. The membranes were incubated in Super-Block blocking buffer (Pierce) for 45 min at room temperature. They were then incubated overnight at 16°C in blocking buffer plus a 1:2000 dilution of a polyclonal sheep antiserum raised to recombinant NL4–3 Nef produced by *Escherichia coli*. Membranes were washed twice for 10 min and once for 20 min in PBS plus 0.1% (vol/vol) Tween 20. After washing, membranes were incubated for 1 h in blocking buffer plus 1:100,000 donkey anti-sheep antibody conjugated to peroxidase (binding site). Immunoreactive product was detected by enhanced chemiluminescence with the ECL Western blotting detection system (Amersham International). Exposure times varied from 45 s to 3 min.

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